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Enhancing Biological Detection With Dendrimer-Based Immunosensors

by Amanda L. Jenkins, Kate K. Ong, and Ray Yin

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Enhancing Biological Detection With Dendrimer-Based Immunosensors

Amanda L. Jenkins, Kate K. Ong, and Ray Yin
Weapons and Materials Research Directorate, ARL

Abstract

Dendrimers are highly branched, tree-like polymers that possess precise size and shape, as well as surface groups. Among them, polyamidoamine dendrimers are the most widely studied water-soluble/biocompatible dendritic macromolecules. Such dendrimers have been exploited for interesting applications in immunodiagnostics, magnetic resonance imaging, gene therapy, and drug delivery. This report introduces a new signal amplification strategy using dendrimers. While only one fluorescein group can be linked onto an antibody molecule, a large number of fluorophores can be attached to an antibody through a dendrimer linker molecule, thus forming a water-soluble fluorescein-dendrimer-antibody bioconjugate. Upon addition of the antigen, enhanced fluorescence signals are obtained when compared with the corresponding fluorescein-antibody analogs.

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1. Introduction

Immunoassays have been widely used in medical diagnostics and environmental sensing [1–4]. Such assays often involve an immunocomplex formation resulting from antibody (Ab)-antigen (Ag) binding reactions. This process, however, does not generate signals that can be readily visualized by the optical microscope. Therefore, a signal generator or a tag molecule (i.e., a fluorescent [Fl] dye) is conjugated onto antibodies for real-time imaging purposes [5]. The sensitivity of this assay often directly correlates with the dye density or the number of dye molecules per antibody. Higher dye density will result in better sensitivity. However, due to the hydrophobic nature of the fluorescent dyes, the attachment of multiple dye molecules onto an antibody often causes severe precipitation problems in buffer. Thus, the sensitivity enhancement based on multiple dye attachment is greatly limited.

On the other hand, polyamidoamine (PAMAM) dendrimers are multifunctional, water-soluble macromolecules that are capable of bearing multiple dyes and antibodies [6–7]. In this report, the fluorescent signals generated from antibody-fluorescein (Ab-Fl) and antibody-dendrimer-fluorescein (Ab-Den-Fl) conjugates, as well as their immunocomplexes with antigens, will be directly compared.

2. Experimental

2.1 Reagents

Polyamidoamine (PAMAM) dendrimers (generation 5, E5) were purchased from Dendritech, Inc., Midland, MI. Anti-bot (bot) toxin Fab and bot toxoid were obtained from the U. S. Army Edgewood Chemical and Biological Center, Aberdeen Proving Ground-Edgewood Area, MD. Fluorescein dye (FITC) was purchased from Sigma. Antibody-FITC, antibody-dendrimer-FITC (A and B), and dendrimer-FITC conjugates were prepared using procedures described previously [8]. The antibody bioconjugate samples were diluted in 10 mM phosphate buffered saline (PBS) pH 7.4 (Sigma Chemical, St. Louis, MO) to a final concentration of 100 μg/mL. The dendrimer-FITC bioconjugate was diluted with 10-mM PBS to a final optical density of 0.6. The antigen (bot toxoid) was diluted to a concentration of 2.5 μg/mL with 10-mM PBS.

2.2 Sample Preparation

Equal volume solutions of bioconjugate (i.e., 100 μg/mL) and bot toxoid (2.5 μg/mL) were mixed, and the resulting mixture was immediately transferred to a microscope slide cleaned with a solution of 0.5-M sodium hydroxide in 25% methanol prior to use. The formation of immunocomplexes was visualized through an optical microscope.

2.3 Instrumentation

Visualization was achieved using an Olympus BX40 (Opelco, Sterling, VA) optical microscope equipped with an epi-fluorescence attachment. The sample spots were excited with a xenon-mercury lamp using a 450-480 nm band pass filter recommended for FITC observation. All observations were made with a 40x objective, and images were captured using a charge-coupled device (CCD) camera (Optronics, Goleta, CA). The images were imported into Adobe Photoshop 5.0, whereby the sample brightness was based on luminosity measurements and the sample size was based on number of pixels. The pixel size was then converted into micron size using an NIST-traceable magnification reference standard (Geller Microanalytical Laboratory, MA) that was imaged under the same conditions as the sample.

3. Results and Discussion

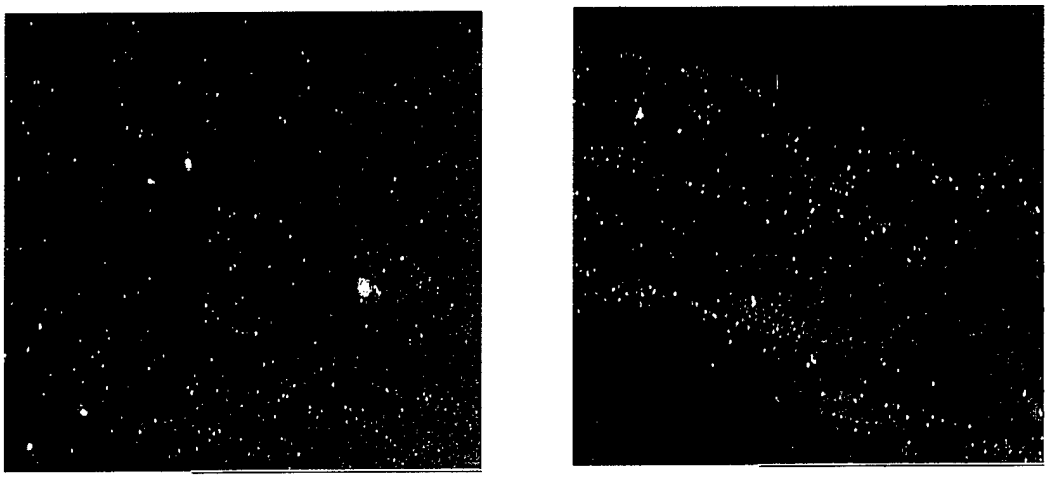
A series of fluorescein-containing bioconjugates was utilized for this study. The ratios of antibody, dendrimer, and fluorescein in different bioconjugates are listed in Table 1.

Table 1. Antibody-dendrimer-fluorescein ratios.

Sample	Ratio of Ab-Den-Fl
Antibody-Fl	1: 0: 1
Antibody-Dendrimer-Fl (A)	2: 1: 10
Antibody-Dendrimer-Fl (A)	1: 1: 10
Dendrimer-Fl	0: 1: 10

3.1 Antibody-FITC Bioconjugate

Antibody-FITC (Ab-Fl) bioconjugates, as well as their immunocomplexes formed with bot toxoid, were directly imaged using a fluorescence microscope, as seen in Figure 1. Aggregates were observed in both cases. The former appeared to be more uniform in size and brightness, while the latter consisted of slightly larger and brighter spots. The luminosity reading obtained by Adobe Photoshop 5.0 measured 210 for the antibody-fluorescein bioconjugate and 233 for the aggregate.



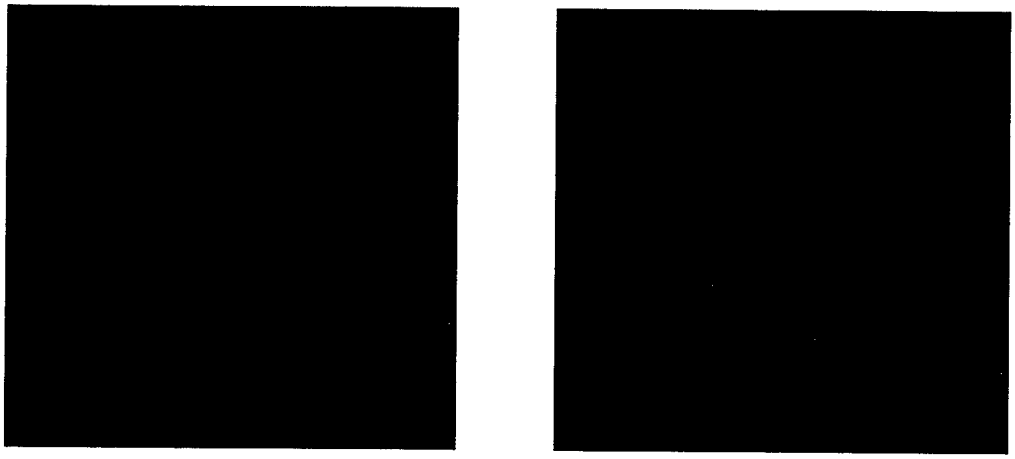
(a) (b)

Figure 1. Fluorescent microscopic images (40×) of antibody-fluorescein bioconjugates (a) without bot toxoid and (b) with bot toxoid.

The stronger background observed in Figure 1(a) was not too surprising, since the introduction of FITC made the Ab-Fl bioconjugates more hydrophobic. When dissolved in PBS buffer, these bioconjugates formed physical aggregates due to the hydrophobic association of the dye molecules. As a result, a micelle-like aggregate with fluorophores at its interior was produced. Such aggregates were very uniform in size, as seen in Figure 1(a). In the presence of bot toxoid, antibodies started to react with multivalent bot antigens, thus forming larger immuno clusters. As indicated in Figure 1(b), these clusters were approximately twofold larger than the physical aggregates. On the other hand, neither PBS buffer (a) nor bot toxoid (b) emitted observable fluorescence in this excitation range, as shown in Figure 2. This eliminated the possibility of background fluorescence and indicated that the larger, brighter spots indeed resulted from the immunocomplex formation.

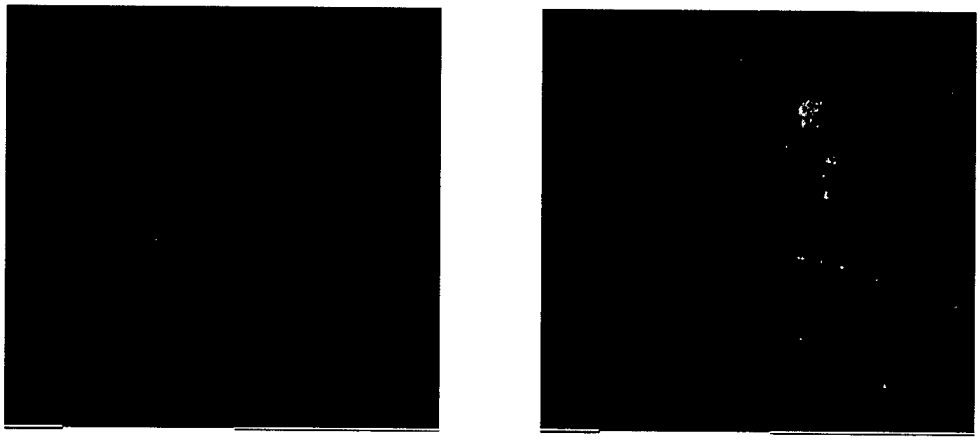
3.2 Antibody-Dendrimer-FITC Bioconjugate

While mantaining the same ratio of the dendrimer and FITC, two paired samples, antibody-dendrimer-FITC (Ab-Den-Fl) bioconjugate A and B, were prepared by varying the number of antibodies on a dendrimer. Ab-Den-Fl bioconjugate A consisted of two antibody molecules per dendrimer, whereas Ab-Den-Fl B only contained one antibody molecule per dendrimer. In the absence of bot toxoid, both bioconjugate A and B formed a uniform film, and no bright aggregate spots were observed, as shown in Figures 3(a) and 4(a). Upon addition of bot toxoid, larger immunoclusters immediately formed in both cases (Figures 3[b] and 4[b]). However, the clusters generated from bioconjugate A appeared noticeably larger and brighter with a luminosity reading of 260 (Figure 3[b]). The luminosity reading measured for bioconjugate B was 240. In both



(a) (b)

Figure 2. Fluorescent microscopic images (40×) of (a) PBS buffer and (b) bot toxoid.

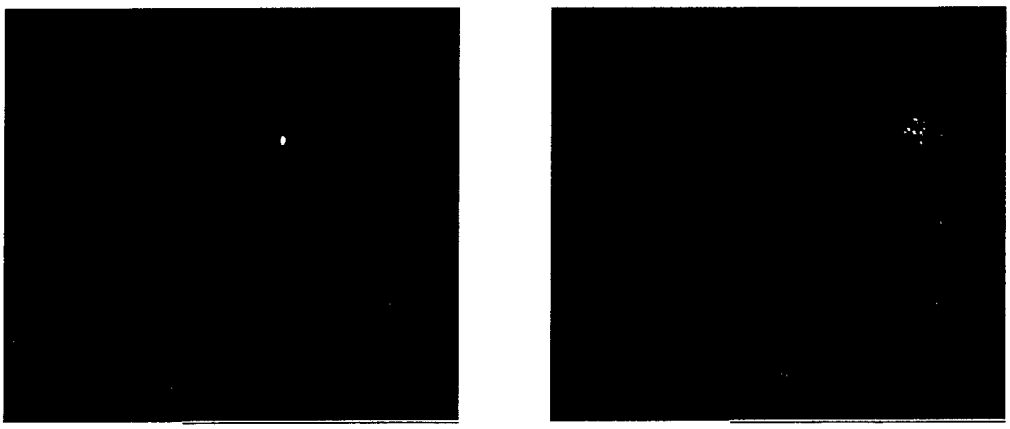


(a) (b)

Figure 3. Fluorescent microscopic images (40×) of antibody-dendrimer-fluorescein bioconjugate A (a) without bot toxoid and (b) with bot toxoid.

cases, the intensity of the fluorescence signals from these clusters was significantly stronger than the corresponding Ab-Fl bioconjugates (Figure 1[b]).

The cleaner background obtained from the Ab-Den-Fl bioconjugates (A and B) suggested that dendrimers were better water-soluble linkers for hydrophobic dyes such as FITC. The addition of a dendrimer linker between organic dyes and antibodies apparently facilitated the dissociation of the physical aggregates or micelles present in the solution. Thus, no bright aggregated spots were observed (Figure 3[a]). Since bioconjugates A and B possessed the same number of FITC groups, the stronger fluorescence intensity observed with bioconjugate A (after



(a) (b)

Figure 4. Fluorescent microscopic images (40×) of ab-dendrimer(B)-fluorescein conjugates (a) without bot toxoid and (b) with bot toxoid.

the addition of bot toxoid) was likely due to the presence of multiple antibodies within the bioconjugate. Typically, two antibody molecules were attached to each dendrimer in bioconjugate A; each bot toxoid exhibited multiple binding sites, highly crosslinked, dense immunoclusters that rapidly formed upon mixing (Figure 5). In contrast, only loose clusters were produced when mixing bioconjugate B with bot toxoid, resulting in much weaker fluoresent signals. The dendrimer linker not only enhanced the dye solubility in water, but also allowed multiple fluorescein and antibody attachment, thus greatly increasing the dye density on the antibody. As a result, the detection sensitivity was significantly enhanced. This was in sharp contrast with the conventional linkers that only allowed a single dye or receptor attachment and did not help to solubilize the resulting bioconjugates.

The bioconjugation process did not alter the antibody performance. Instead, signal enhancement was obtained from bioconjugates containing multiple dyes and antibodies.

3.3 Dendrimer-FITC Bioconjugates

To demonstrate the possibility of nonspecific binding occurrence, dendrimer-FITC bioconjugate samples were tested alone and in the presence of bot toxoid. The dendrimer-FITC bioconjugate itself fluoresced brightly; however, no aggregates were observed. When an equal volume solution of bot toxoid was added to the sample, no aggregates were observed.

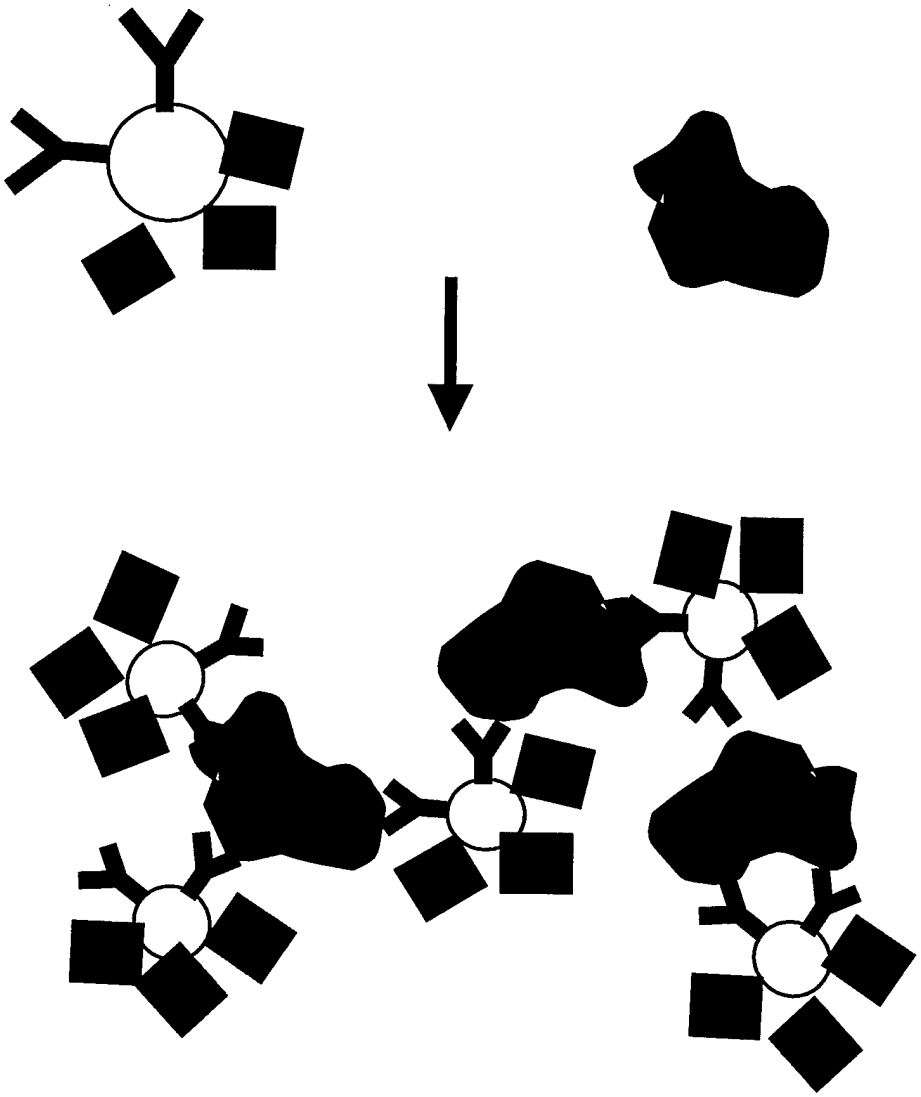


Figure 5. Upon reaction with antibody (red)-dendrimer (circle)-fluorescein (green) bioconjugate A and the bot toxoid (blue), highly crosslinked, dense immunoclusters formed immediately.

This result suggested that in the absence of anti-bot toxoid Fab, dendrimer-FITC conjugates alone were not capable of reacting or interacting with the bot toxoid, as evidenced by the absence of immunoclusters. Therefore, the antibody-antigen reactions were the main cause for the formation of immunoclusters, and the nonspecific binding was not present between the dendrimer and the bot toxoid.

4. Conclusions

Visualization of Ab-Den-Fl bioconjugates as well as their immunoclusters has been successfully achieved using fluorescence microscopy. Using this technique, the immunoreactions between anti-bot-dendrimer-FITC bioconjugates and the bot toxoid can be directly studied and imaged in real time. Bioconjugates containing multiple dyes and antibodies tend to give more intense fluorescence signals, thus better assay sensitivity. Non-specific binding is not observed between the dendrimer-FITC bioconjugate and the bot toxoid. Multifunctional linkers such as dendrimers can be used as a general signal amplification strategy for immunoassays and other receptor-based assays.

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